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Immunosensing with artificial antibodies in organic solvents or complex matrices

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ABSTRACT

The detection of analytes in complex matrices without labour intensive sample preparation is an important goal in analytical chemistry. In this article we would like to address this issue by transferring the selectivity of natural antibody in a cheap, robust and reusable polymer, employing a double imprinting protocol. Antibodies with the desired selectivity were used as template to generate imprinted polymer particles. These antibodies were added to a prepolymer and particles were precipitated. After the antibodies were removed from the particles, cavities remained which reproduce size, shape and surface chemistry of the antibodies. In a second imprinting step the particles with cavities were pressed into a second polymer. After the second polymer has cured the particles can be removed leaving positive structures behind that react with the desired antigen. Such a sensitive coating was applied to the surface of a quartz crystal microbalance and incorporated into a microfluidic chip. An immunosensor for estradiol was fabricated having six times higher affinity to its antigen than to structurally related molecules. The measurements can also be performed after an extraction into an organic solvent which improves the detection limit greatly and would not be possible for natural antibodies. The feasibility of the method for complex matrices was shown by detecting viruses in plasma or allergenic protein in bread extract.

1. Introduction

Immunosensing has become a standard technique for all kinds of different analytes. Molecularly imprinted polymers (MIPs) are an interesting alternative since they are more robust and cheaper as their natural counterparts. Such polymers are successfully used as stationary phases in chromatography [1,2], as catalysts [3], as drug delivery vehicles [4], to induce crystallization [5,6] or as sensor materials [7–9]. MIP based selective materials have been developed for analytes ranging from ions [10] or small molecules [11–14], proteins [15], viruses [16] to even entire cells [17]. While excellent selectivities have been achieved for small molecules [18], fabricating reliable MIPs for biomolecules is still an issue. One main problem in imprinting with biomolecules is that the standard imprinting approach (a template molecule is simply added to the prepolymer [19,11]) does not work due to large molecule sizes. Large molecules would simply be irreversibly trapped into the polymer. To address this problem, surface imprinting was developed [20]. Using imprinted nanoparticles is one approach to increase the surface area of the imprinted polymer [21–23]. Recently, methods for double imprinting were developed to transfer the selectivity of natural antibodies or enzymes to a polymer [24–27] (see Fig. 1).

To this end, nanoparticles are printed with antibodies. The polymer is allowed to cure and the particles are washed to remove the antibody. These nanoparticles are adhered on a stamp and used for a second imprinting process leading to a polymeric antibody copy. Compared to previously shown surface imprinting methods, double imprinting leads to a significantly increased surface and thus more binding sites. Furthermore, the recognition mechanism is different than in conventional imprinting. In contrast to conventional imprinting where the whole template is recognized, double imprinted surfaces (as natural antibodies) recognize epitopes (substructures of a molecule) [28]. This is believed to be favourable for recognition of large and complex biomolecules [29]. This method has so far only been used for aqueous solutions. In this article we extended the method to different analytes as well as more relevant matrices as plasma or food samples. We employ the principle of polymeric antibody copies to detect the following bioanalytes: sesame protein, which has been recognized as an increasingly frequent and potentially serious allergen [30] and estradiol and its structural analogues, which are serious pollutants due to their influence on the hormonal system [31]. Furthermore, we present a new way to pre-concentrate and measure hormons in an organic solvent. It has to be noted that some authors also call imprinting with two different template molecules double imprinting [32]. In contrast the presented technique consists of two imprinting steps instead of one step with two templates.

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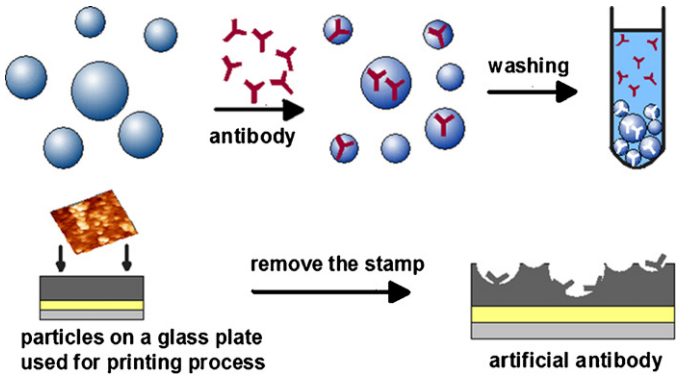

Fig. 1. Principle of artificial antibodies. A prepolymer is precipitated in presence of natural antibody to obtain imprinted nanoparticles. The immunoglobulin is washed out by centrifuging and the resulting particles are adhered on a stamp. The stamp is pressed into a prepolymer on a quartz microbalance and the polymer is allowed to cure. When the stamp is removed, a copy of the natural antibody remains.

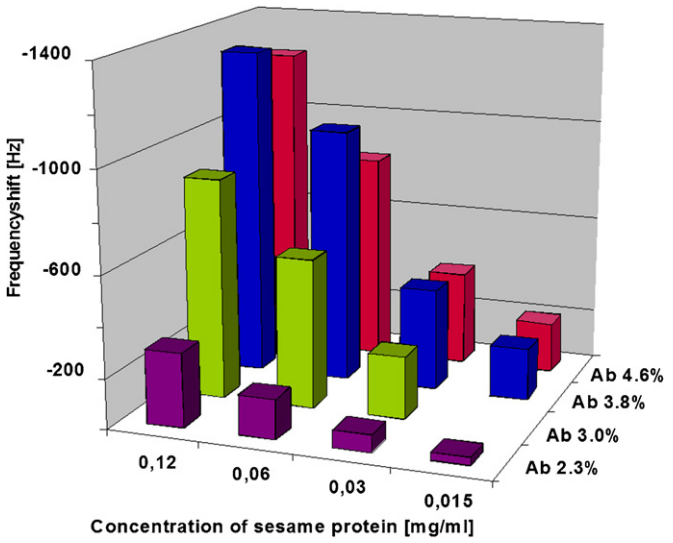

Fig. 2. Optimization of antibody(Ab) consumption. Sensors were produced for each concentration and the response to the antigen (sesame protein in this case) at different concentrations was determined. 3.8% of the polymer mass was found to be ideal.

2. Experimental details

2.1. Materials

We purchased all chemicals in analytical grade or highest synthetic purity from Fluka, Merck and Sigma Aldrich. Commercial food samples were taken from local shops. Food protein extracts were prepared by soxhlet extraction from food samples as shown elsewhere and the concentration was determined by a Bradford assay [33]. 10 mg of food sample were grinded thoroughly and extracted with 50 ml of n-hexane for 18–20 h. The sample was dried over night and diluted in PBS. The samples were centrifuged and the supernatant was taken. The protein concentrations were tested photometrically by Bradfordtest. Human rhinoviruses were generously donated by the group of Prof. Blaas (University of Vienna). We obtained blood samples that served as a matrix for virus sensing from the Austrian Red Cross. The only sample preparation that was performed on the blood samples was a centrifugation to remove the majority of the blood cells. Anti sesame protein immunoglobulin Y from eggs laid by immunized chicken was extracted according to the procedure published by McKinney and Parkinson [34]. Anti estradiol, anti-rhinovirus and anti insulin antibodies (purified monoclonal) were purchased from Santa Cruz Biotechnology. For microfluidic chip fabrication the elastomer kit from sylgard was used.

2.2. Double imprinting

For measurements with antibody copies, imprinted particles were synthesized. To this end, 50 mg methacrylic acid, 20 mg vinylpyrollidone and 60 mg dihydroxyethylenebisacrylamide (DHEBA) are dissolved in 800 μl of water at 70 °C. The solution is neutralized (to a pH of 7) to retain the antibodies natural conformation and 1.5 mg sodiumperoxydisulfate are added to start the reaction. During that step the prepolymer also has time to cool to room temperature. After mixing the solution thoroughly different amounts of natural antibody (3.8% is optimal) are included and the mixture is pre-polymerized under UV-light for 30 min. The prepolymer (20 μl/ml) is dropped into acetonitrile during fast rotation and stirred over night. Complexes between the antibody and the nascending polymer are successively formed by self-assembling. Thus, it is crucial to choose monomers that complement the chemical moieties on the template molecules [35,36]. In the presented case, we believe that the main binding mechanism is governed by the formation of hydrogen bonds and by hydrophobic interactions. This is in agreement with what has been reported for different biosamples [37–39]. At the same time the polymer is crosslinked which guarantees that the polymeric cavity preserves the shape of the antibody. The solution is centrifuged at 2000 rpm for 5 min and the acetonitrile is removed. The presence of the immunoglobulin in the pellet was detected by non-specific protein labelling with dansylchloride [40]. To remove the antibody from the cavities the pellet is redispersed in distilled water. After another centrifugation (2000 rpm for 5 min) the antibody can be detected in the supernatant. The washing procedure is repeated twice and the absence of the antibody in the pellet is verified by labelling with dansylchloride. After drying the stamp is pressed into a polymer (same composition as the polymer for particles but with 30 mg of DHEBA instead of 60 mg and 1:2 diluted in water) on the measuring electrode of a QCM. The reference electrode is also coated with polymer and printed with the non imprinted particles.

2.3. Measuring setup

For the QCM-measurements we use dual electrode geometry (one measuring and one reference electrode). These structures were screen printed with gold paste (from Heraeus) onto quartz discs (10 MHz, AT-cut, 15.5 mm diameter) and burned at 400 °C for 3 h. Electrodes oriented towards the aqueous phase are grounded and have 5 mm in diameter whereas electrodes oriented to the gas phase are 4 mm in diameter. To minimize the length of diffusion paths (and thus reducing sensor response times) the quartz sensor is incorporated into a microfluidic chip. To this end, the quartz plate and the connecting electrodes are sandwiched between two PDMS layers. Both are equipped with a measuring chamber placed right on top or underneath the electrodes. The top layer has an inlet and an outlet whereas the bottom layer only consists of a chamber that is filled with air and allows oscillation of the quartz sensor.

3. Results and discussion

3.1. Preliminary control experiments

An important preliminary parameter is the amount of antibody starting material that is used. Fig. 2 shows that 5 mg which corresponds to 3.8% leads to the highest sensor responses and thus is the optimal antibody concentration.

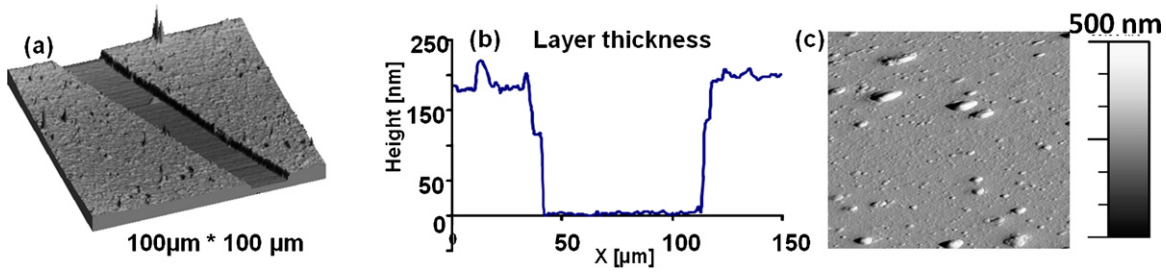

Fig. 3. (a) AFM-scan (recorded with a Digital Instruments IIIA system in contact mode) of a scratch to determine the polymer thickness, (b) cross section taken perpendicular to the scratch and (c) AFM image of the imprinted nanoparticles (5 μm × 5 μm).

Since the thickness of the polymer coating is crucial for the success in QCM measurements it was determined by atomic force microscopy.

As shown by scanning a scratch in the polymer in Fig. 3, the thickness of the polymer coating is around 200 nm. That height corresponds to a little bit less than half of the average size of the particles (around 500 nm) and was found to be optimal. If the thickness is increased, particles sink too deep into the polymer and cannot be removed anymore. Lower thicknesses lead to less recognition surface and thus lower sensor responses. Furthermore, the size of the nanoparticles, which were typically a few hundred nm in diameter, was confirmed by AFM.

3.2. Detection of hormons

Fig. 4a shows a typical QCM-measuring curve obtained with an artificial immunosensor for estradiol. First, the system is filled with water (or whatever other solvent or buffer the analyte is in) to measure the baseline. Injection of estradiol leads to a drop in frequency which is proportional to the mass that is detected by the sensor (the dependency is shown in Fig. 4b).

When the analyte solution is replaced by water the resonance frequency returns to its previous value. It has to be noted that this is a big advantage over the use of natural antibodies where regeneration is either not possible or strong chaotropic substances

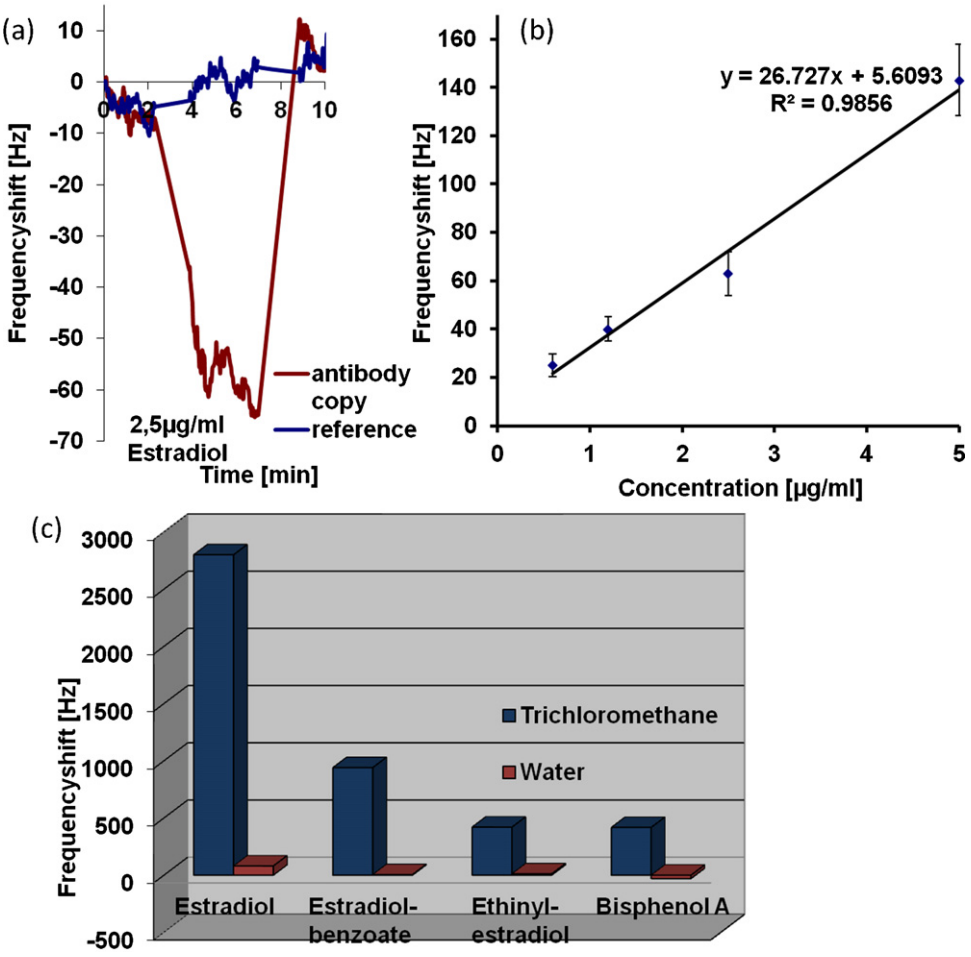

Fig. 4. (a) A typical measuring curve detected with a 10 MHz quartz equipped with a measuring electrode and a reference. Insertion of the template estradiol results in a drop in the resonance frequency. To recover the sensor water is injected again, (b) sensor characteristic for the measurement of estradiol and (c) comparison of measurements of estradiol and potentially cross reactive substances in water and after an extraction with trichloromethane (measured in the organic phase).

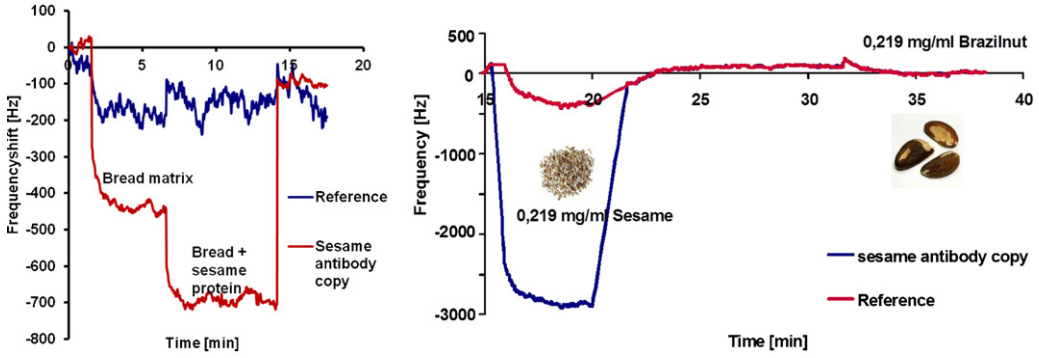

Fig. 5. (Left) QCM-measurement (measuring electrode and reference) of commercial whole grain bread samples spiked with sesame (0.007 mg/ml). (Right) The quality of the sensor can be discovered by a QMB-measurement of sesame protein and other potentially cross reactive proteins. Therefore, sesame and brazilnut extracts are measured in the same concentrations.

are required for washing [24]. The detection limit (500 ng/ml) was improved by a factor of ten by introducing an extraction step before sensing. Therefore, the aqueous solution of the hormone is mixed with a small amount of chloroform (1 ml/10 ml of the aqueous solution) in a separation funnel. Consequently, the analyte can be measured in the organic phase where it is preconcentrated which would not be possible with natural antibodies since they are denatured in organic solvents. However, the PDMS based chip cannot be used for organic solvents so the quartzes were directly inserted into the solution. As baseline chloroform from an extraction funnel with pure water was used to exclude unspecific effects from higher water-content. The obtained sensor responses in organic and aqueous phase as well as for structural analogues are depicted in Fig. 4c. Bisphenol A which showed the lowest cross reactivity and is the least similar molecule, was also tested since it is known to affect the hormone system.

3.3. Detection of sesame protein

To test the feasibility for sensing in complex matrices we chose to detect allergenic sesame protein in bread samples. As a preliminary test, the sensors ability to differentiate between different protein extracts was tested and one of those tests is shown in Fig. 5 (right).

Besides brazilnut also rye and sunflower protein were tested and showed nearly no cross reactivity. A selectivity factor of 5 was found for wheat protein. The left side of Fig. 5 shows the actual experiment for detection of sesame in whole grain bread. Since other bread components were present in excess in the sample, the matrix causes a signal shift as well. However, compared to the bread sample, insertion of the spiked one still leads to a frequency drop. Fig. 6 shows the sensor characteristic for sesame detection.

The error bars are the standard deviations determined from three different independent measurements. An additional control experiment was performed in order to exclude, that the selective sensor response is due to an unknown high affinity between the polymer to the analyte. Artificial reference antibodies for insulin were prepared and the response of polymeric antibodies to insulin and sesame protein was tested.

It can be seen in Fig. 7 that both artificial antibodies bind their respective antigen significantly stronger than the other protein. This finding supports that the selective sensor response that was found is due to a transfer of selectivity from the natural antibody to the polymer.

3.4. Detection of viruses in plasma

In analogy to the measurements shown before artificial antibodies for the detection of human rhinovirus (HRV) were created.

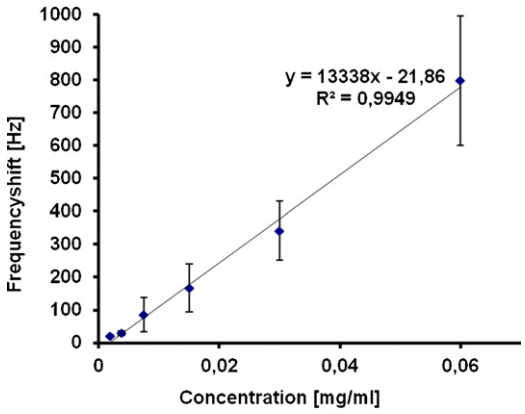

Fig. 6. Sensor characteristic for measurement of sesame protein in water reaching a detection limit of 0.002 mg/ml.

Since rhinovirus was stored in phosphor buffered saline (PBS) we used PBS to determine the baseline in all measurements of HRV.

Fig. 8 shows the measurement of HRV in plasma. Similar to the measurements shown before it is also possible to measure a frequency response from the virus in a complex and not exactly known matrix. The lowest detected concentration of virus in plasma was 10^{14}/ml.

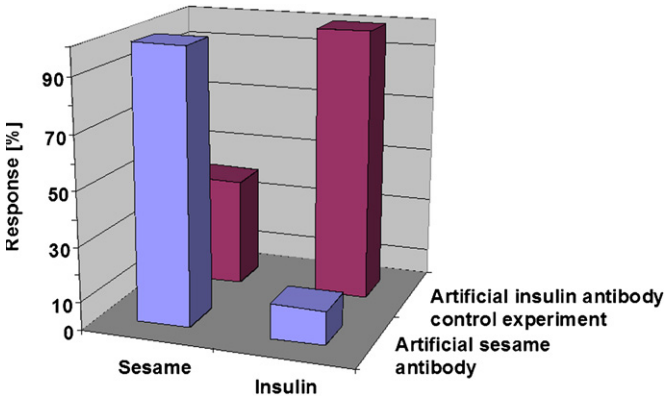

Fig. 7. Control experiment to confirm that antibody selectivity was transferred to the polymer. Artificial insulin antibodies were prepared in the same way as artificial sesame protein antibodies. The sensor responses and cross reactivities were tested for both proteins.

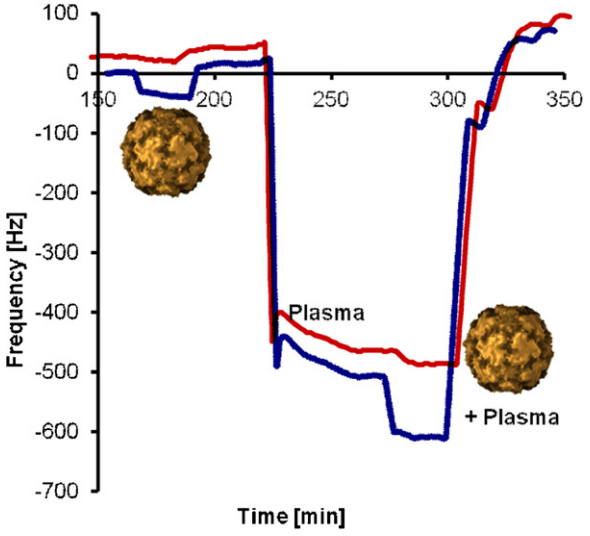

Fig. 8. Sensing human rhinovirus in plasma. The measuring electrode is shown in blue the reference is shown in red. The first frequency response was caused by the injection of 10^{15} viruses/ml in PBS (PBS buffer pH 7.4 was used as baseline). After confirming, that the sensor response is reversible, the matrix (plasma + the same amount of PBS the virus was contained in but no virus) is inserted leading to a frequency drop. When a spiked sample is added the frequency drops further.

4. Conclusions

The measurement with artificial antibodies turned out to be a powerful method to detect bioanalytes. Selectivity of antibodies can be combined with robustness of artificial polymers. While a conventional immunosensor can usually only be used once or at least is limited by the lifetime of the antibody (about 2 weeks). Compared to natural antibodies attached to directly attached to the gold electrodes the artificial ones usually have slightly worse selectivities. Sensitivity is usually higher since the double imprinting process leads to a larger surface and thus more binding sites (tested for sesame protein and virus). The artificial ones can be used by an order of magnitude longer and under non biocompatible conditions. It is possible to measure in complex matrices as food samples or plasma without knowledge of the exact composition. Artificial antibody copies are an alternative for direct imprinting if the analyte is unstable and will denature during the imprinting process. Additionally, instead of the whole analyte that is recognized in conventional imprinting, artificial antibodies have an epitope which generally leads to less cross reactivity in substructure imprinting [29].

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